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Yamamoto et al., Nature 319: 230-234 1986

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Thanks,

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Inhibition of Tyrosine Kinase Activity of the Epidermal Growth Factor (EGF) Receptor by a Truncated Receptor Form That Binds to EGF: Role for Interreceptor Interaction in Kinase Regulation

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The tyrosine kinase activity of the epidermal growth factor (EGF) receptor is regulated by a truncated receptor of 100 kilodaltons (kDa) that contains the EGF-binding site but not the kinase domain. The inhibition of kinase is not due to competition for available EGF or for the kinase substrate-binding site. Chemical cross-linking studies suggest that the 100-kDa receptor may form a heterodimer with the intact EGF receptor. Structurally related receptor kinases, such as the platelet-derived growth factor receptor, the insulin receptor, and the Neu receptor, were not inhibited by the 100-kDa receptor. The results indicate that (i) the inhibition was specific for the EGF receptor, (ii) the kinase domain had little or no role in determining target specificity, and (iii) the regulation of kinase may be due to a specific interaction of the 100-kDa receptor with the ligand-binding domain of the EGF receptor kinase.

The epidermal growth factor (EGF) receptor (9) is a plasma membrane protein that represents the product of a gene related to the v-*erbB* proto-oncogene. Its cytoplasmic domain shows extensive homology with the tyrosine kinase family of proteins. Tyrosine kinases are intimately involved in cellular growth control and oncogenesis. Their importance is apparant in the stringent conservation of these molecules through prolonged evolutionary times and in the remarkable homologies seen between different tyrosine kinases in their catalytic domains (9, 13). Recent in vitro mutagenesis experiments have demonstrated a requirement for intrinsic tyrosine kinase in the actions of the EGF receptor (10, 12).

The EGF receptor, a single-chain polypeptide of 170 kilodaltons (kDa), belongs to a unique class of bipolar membrane proteins in which a single membrane-spanning segment separates the extracellular (ligand-binding) domain from the intracellular (tyrosine kinase) domain (2, 11). Two types of models can be proposed for kinase-activating signal transduction across the membrane. In the first model, it is assumed that an EGF-induced conformational change in the extracellular domain is transmitted through the single 23-residue-long transmembrane region to the kinase site, which is consequently activated. According to the second model, the kinase site is regulated by interreceptor association-dissociation events, and EGF activates the kinase by influencing the association equilibrium. There is experimental evidence in support of both models (5, 7, 8, 14, 18, 24). The monomeric receptor has been found to be the catalytically reactive form by us and others (5, 7, 14, 18). According to some workers (14, 18), the monomeric receptor molecule has the potential to function as a transmembrane signal transducer, whereas our sedimentation and kinetic data indicate that the EGF receptor interconverts between an active monomeric state and a noncovalently linked dimeric form (5, 7). Other workers have also found the occurrence of dimeric EGF receptors but have proposed that dimerization serves to activate rather than regulate the kinase site (8, 24).

Some of the questions raised by the monomer-dimer model are (i) whether truncated EGF receptors can form stable dimers and (ii) whether a truncated receptor can form a heterodimer with the intact receptor and influence its kinase function. A truncated and oncogenic homolog of the EGF receptor, the 68-kDa v-*erbB* protein retains the kinase domain and the membrane-spanning segment but not the EGF-binding domain. It is a constitutively active tyrosine kinase. The counterpart of this kinase is a 100-kDa receptor (15, 22) that contains the EGF-binding site but lacks the membrane-spanning segment and the kinase domain. In earlier studies, we tested these truncated proteins and other similar proteins generated in vitro (2, 3) for dimer-forming ability and found that they lack the ability to form stable homodimers (5). Thus, both the external and cytoplasmic domains of the receptor must be present to contribute to the tight binding necessary for dimer formation. In this communication, we report on the ability of the isolated external domain to inhibit the kinase function of the intact EGF receptor specifically. The 100-kDa receptor had no effect on the kinase activities of related growth factor receptors, implying that the structural specificity for inhibition lies not in the kinase domain but in the external domain of the target receptor kinase. However, the inhibition is not a simple consequence of competition for available EGF. Overall, the results support a role for interreceptor association in kinase regulation.

MATERIAL AND METHODS

Isolation of the 170-kDa EGF receptor. Plasma membranes were isolated from A431 cells, and the 170-kDa EGF receptor was solubilized and purified as previously described (4, 7). The amount of the receptor present in membranes or in purified preparations was estimated by Scatchard analysis of [^{125}I]EGF-binding data based on a 1:1 complex of EGF and the receptor (7).

Isolation of the 100-kDa EGF receptor. The 100-kDa receptor was purified from conditioned medium of A431 cells. Cells (10^7) were seeded into 15-cm-diameter dishes in Dulbecco modified Eagle medium containing 7% fetal bovine serum. After 24 h at 37°C, the cells were washed with

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phosphate-buffered saline and then incubated at 37°C for 24 h with serum-free Dulbecco modified Eagle medium. The conditioned medium was collected, centrifuged to remove cellular debris, and concentrated by ultrafiltration on PM-30 filters, and the buffer was adjusted to 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.4)–10% glycerol, 50 μg of leupeptin per ml–2 mM phenylmethylsulfonyl fluoride (PMSF). The concentrated conditioned medium (300 μl from 24 dishes) was stirred at 4°C for 1 h with 100 μl of packed WGA-Sepharose. The gel pellet was washed six times with 20 mM HEPES (pH 7.4), and the 100-kDa protein was eluted by stirring the gel at 4°C for 1 h with 1 ml of 0.6 M *N*-acetylglucosamine in the HEPES-glycerol-leupeptin-PMSF buffer. The eluate was concentrated by ultrafiltration, and the buffer was adjusted to 20 mM HEPES (pH 7.4)–10% glycerol–10 μg of leupeptin per ml–1 mM PMSF–0.2 mM sodium vanadate. The amount of the 100-kDa receptor present was estimated by [^{125}I]EGF-binding data based on a 1:1 complex of EGF and the receptor.

Isolation of insulin and PDGF receptors. The insulin receptor was purified from human placental membranes as previously described (20). Highly purified platelet-derived growth factor (PDGF) receptor was isolated from human MG-63 cells (6).

Antibodies and other reagents. Mouse monoclonal anti-EGF-receptor antibody 425, used for the preadsorption experiment, was generated as previously described (2, 16). The polyclonal anti-EGF receptor antibody generated in rabbits was used for immunoblotting as previously described (17). The chemical cross-linking reagents were purchased from Pierce Chemical Co.

RESULTS

Inhibition of tyrosine kinase activity of the EGF receptor by the 100-kDa EGF receptor. The 100-kDa EGF receptor was isolated from conditioned medium of A431 cells. Coomassie blue staining of the purified preparation after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed a single protein band at ~100 kDa which showed reactivity with anti-EGF receptor antibody and which could be affinity labeled with [^{125}I]EGF (Fig. 1). The K_d for binding to [^{125}I]EGF was ~100 nM (data not shown).

The purified preparation of the 100-kDa receptor was tested for its effect on EGF receptor kinase activity. The 100-kDa protein inhibited the basal and EGF-dependent autophosphorylating activities of the membrane-bound EGF receptor from A431 cells (Fig. 2A). Other studies showed that the 100-kDa protein also inhibits the autokinase activity of the human placental membrane EGF receptor (data not shown), indicating that inhibition occurs independently of the tissue origin of the EGF receptor.

We next tested the EGF–Affi-Gel-purified 170-kDa EGF receptor for inhibition by the 100-kDa receptor. This purified 170-kDa receptor form showed high basal activity, i.e., high kinase activity, even in the absence of EGF (7). Greater than 90% of the basal autokinase activity was inhibited in the presence of 2.5 pmol of the 100-kDa protein (Fig. 2B). A similar inhibition of autokinase activity was seen with the WGA-Sepharose-purified 170-kDa receptor (Fig. 2C). Both basal and EGF-stimulable autophosphorylations were inhibited, indicating that the inhibition is not a simple consequence of reduction in available EGF. It should also be noted that under the experimental conditions used for Fig. 2A and C there was a high excess of EGF over the EGF-

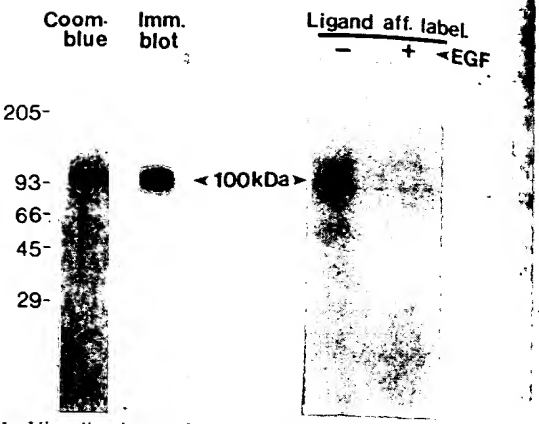

FIG. 1. Visualization of the 100-kDa EGF receptor by Coomassie blue staining, immunoblotting, and covalent linkage to [^{125}I]EGF. Lanes 1; Coomassie blue staining of 50 pmol of the protein after SDS-PAGE; 2, Immunoblot analysis of the protein preparation (~2 pmol) with a rabbit polyclonal anti-EGF receptor antibody as previously described (17); 3 and 4, ligand affinity labeling of ~1 pmol of the protein with 100 nM [^{125}I]EGF as previously described (16) in the absence (lane 3) or presence (lane 4) of excess (3 μM) unlabeled EGF. The numbers to the left indicate molecular sizes in kilodaltons.

binding receptor forms. This discounts the likelihood that competition for available EGF caused the inhibition.

Studies on the time course of the effect showed that maximal inhibition was achieved within 5 min of pretreatment of EGF receptor kinase with the 100-kDa protein (Fig. 3A). When the time of pretreatment with the 100-kDa protein was kept constant (10 min) and the time of the phosphorylation reaction was varied (2 and 15 min), there was no change in the degree of inhibition, i.e., the fractional extent of inhibition was identical for both incubation periods (Fig. 3B).

The loss of receptor autokinase activity described in Fig. 2 and 3 could be due to inactivation of either the kinase catalytic site or the phosphate acceptor tyrosine sites. To distinguish between these alternatives, loss of kinase activity was measured by using an exogenous synthetic peptide substrate (7, 19) which contains no serine or threonine and is a substrate only for tyrosine kinases. The results in Fig. 4 demonstrate loss of synthetic-substrate-phosphorylating activity in the presence of the 100-kDa protein. Thus, the loss of activity was due to inhibition of the kinase site.

To test the effect of the 100-kDa protein on the kinetic parameters of the kinase reaction, we measured initial rates of autophosphorylation over a range of ATP concentrations (0.3 to 30 μM). The V_{max} of the autokinase reaction was reduced by ~50% in the presence of 500 fmol of the 100-kD protein, but the affinity (K_m) for ATP was unchanged (Fig. 4A). To test for any change in affinity for the tridecapeptide substrate, we measured initial rates of phosphorylation over a range of concentrations of the tridecapeptide substrate (0.5 to 5 mM), keeping the ATP concentration at saturation (15 μM). The 100-kDa protein reduced the V_{max} of the phosphorylation reaction, but the affinity of the receptor kinase for the tridecapeptide substrate (K_m, 1.5 to 2 mM) was unchanged (Fig. 4B).

Overall, the results (Fig. 2 to 4) demonstrated that the 100-kDa protein inhibited the kinase activity of the 170-kD EGF receptor irrespective of whether the receptor was membrane bound, solubilized, or purified. The basal and

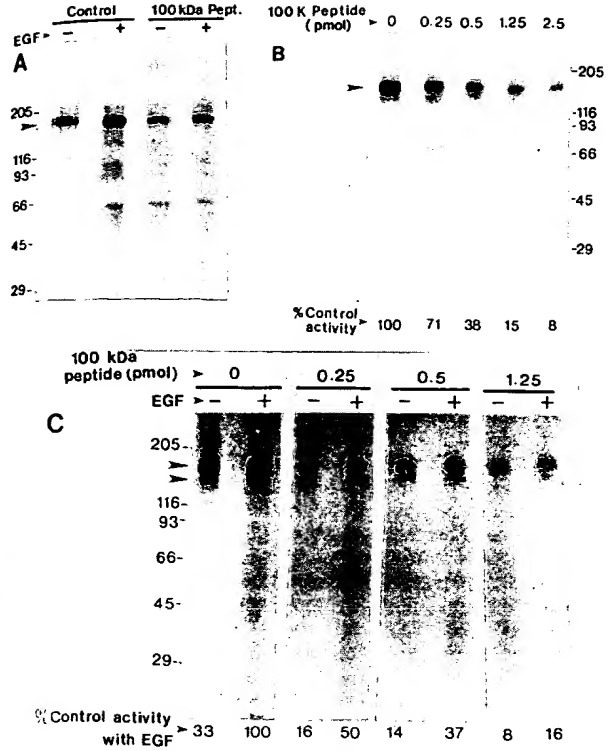

FIG. 2. Inhibition of EGF receptor kinase activity by the 100-kDa soluble secreted EGF receptor. (A) Inhibition of autokinase activity of the plasma membrane-bound 170-kDa EGF receptor (~40 fmol of the receptor) by the 100-kDa receptor (500 fmol). (B) Inhibition of basal autokinase activity of the EGF–Affi-Gel-purified EGF receptor (70 fmol). (C) Inhibition of basal and EGF-stimulated autokinase activities of the WGA-Sepharose-purified EGF receptor (70 fmol). To test for inhibition of autophosphorylation of purified receptors by the 100-kDa receptor (B and C), the 170-kDa receptor (70 fmol of either EGF–Affi-Gel-purified or WGA-Sepharose-purified material) was incubated at 4°C for 10 min with 0 to 2.5 pmol of the 100-kDa protein in 15 μl of a solution containing 20 mM HEPES (pH 7.4), 10% glycerol, 0.1% Triton X-100, 10 μg of leupeptin per ml, 1 mM PMSF, and 0.2 mM sodium vanadate. The mixture was then incubated at 4°C for an additional 10 min in the presence or absence of 1 μl of 40 μM EGF. Phosphorylation was initiated at 20°C by addition of 5 μl of 60 μM [γ-^{32}P]ATP (20,000 cpm/pmol) containing 4 mM $MnCl_2$. After incubation at 20°C for 15 min, the reaction was terminated and the mixture was analyzed by SDS-PAGE and autoradiography as previously described (7). In experiments with the membrane-bound EGF receptor (A), A431 membranes (2 μg of protein; ~40 fmol of the EGF receptor) were treated with the 100-kDa peptide (500 fmol) and EGF exactly as described for the solubilized and purified receptor, except that Triton X-100 was omitted. Initiation of phosphorylation with [γ-^{32}P]ATP, its termination, and its analysis by electrophoresis and autoradiography were the same as those for the solubilized receptor. Receptor phosphorylation was quantified by densitometric analysis of the autoradiograms. The numbers to the sides indicate molecular sizes in kilodaltons.

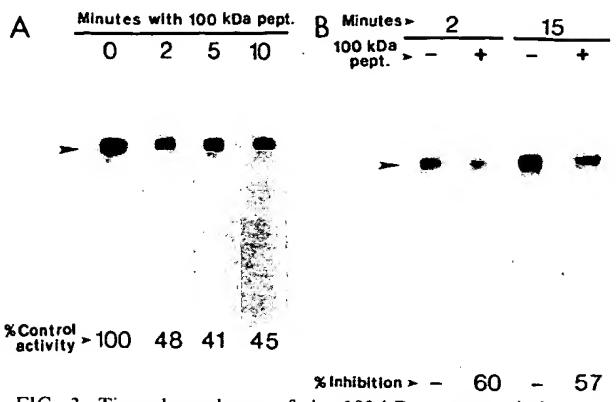

FIG. 3. Time dependence of the 100-kDa receptor-induced effect. (A) Effect of time of incubation of the 170-kDa receptor with the 100-kDa protein. (B) Effect of time of incubation with [γ-^{32}P]ATP. For the experiment whose results are shown in panel A, the EGF-Affi-Gel-purified 170-kDa receptor (70 fmol) was incubated with the 100-kDa protein (500 fmol) at 4°C for the indicated times and then assayed for autokinase activity as described in the legend to Fig. 2. The extent of kinase inhibition was determined by densitometric analysis of the autoradiograms. For the experiment whose results are shown in panel B, the EGF-Affi-Gel-purified 170-kDa receptor (70 fmol) was incubated with the 100-kDa protein (500 fmol) at 4°C for 10 min as described in the legend to Fig. 2. The kinase reaction was started by addition of [γ-^{32}P]ATP (final concentration, 15 μM) and $MnCl_2$ (final concentration, 1 mM). After incubation at 20°C for the indicated times, the reaction was terminated and the products were analyzed as described in the legend to Fig. 2. The inhibition was quantified by densitometric analysis of the autoradiograms.

EGF-stimulated kinase activities were inhibited. The results (Fig. 2 and 3) also demonstrated that the 100-kDa protein itself was not a substrate for the EGF receptor kinase. These results and the kinetic data in Fig. 4 discount the likelihood that the inhibition of kinase activity was due to competition for the substrate-binding site of the 170-kDa receptor kinase.

Inhibition of kinase activity was not due to a contaminating phosphatase or proteinase. We next tested whether the observed inhibition of kinase activity was due to a contaminating phosphatase. The ^{32}P-labeled receptor (EGF–Affi-Gel purified) was separated from unreacted [γ-^{32}P]ATP by ultrafiltration and then incubated with the 100-kDa protein in the presence of 15 μM unlabeled ATP. The ^{32}P-labeled receptor did not dephosphorylate to any noticeable extent over a period of 4 h, indicating that the observed inhibition of kinase was not due to an enhanced rate of dephosphorylation (data not shown).

To test for the presence of a contaminating proteinase, the EGF–Affi-Gel-purified 170-kDa receptor was incubated with the 100-kDa protein for up to 4 h and then tested for any decrease in the immunoreactive 170-kDa protein by Western blotting (immunoblotting). There was no decrease in the amount of the immunoreactive 170-kDa receptor, even after 4 h of exposure to the 100-kDa protein (data not shown). These results indicate that the inhibition of kinase activity was not due to proteolysis of the receptor polypeptide.

Preadsorption with a specific antibody abolished the inhibitory activity of the 100-kDa receptor. The 100-kDa receptor is recognized by external-domain-specific anti-EGF receptor antibodies (2, 16, 17). To test unequivocally whether the 100-kDa receptor itself is causal for kinase inhibition, we preadsorbed the protein preparation with a specific monoclonal antibody. Monoclonal antibody 425 is directed to a polypeptide epitope present in the external domain of the EGF receptor (2, 16) and recognizes both the 170-kDa and the 100-kDa forms of the receptor. Preadsorption of the 100-kDa protein preparation with this antibody resulted in complete loss of inhibitory function (Fig. 5). Thus, the immunoreactive 100-kDa EGF receptor is responsible for the inhibition of kinase.

The 100-kDa protein did not inhibit the autokinase activities

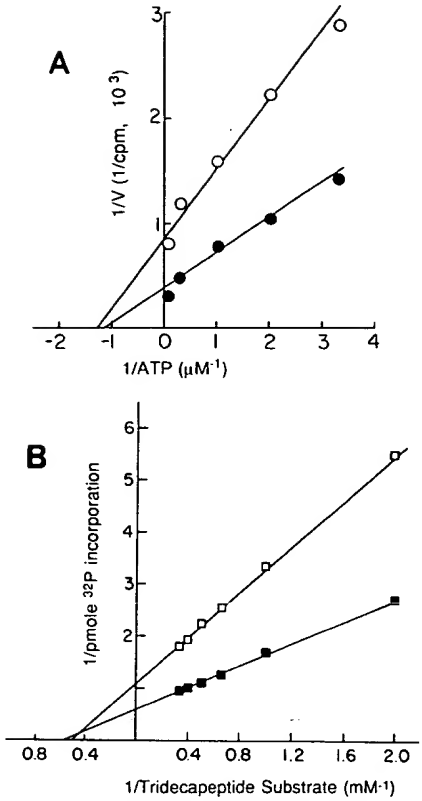

FIG. 4. Effect of the 100-kDa receptor on the V_{max} and substrate affinity of the EGF receptor kinase. (A) Effect of the 100-kDa receptor on the affinity of the kinase for ATP. (B) Effect of the 100-kDa receptor on the affinity of the kinase for a synthetic tridecapeptide substrate. Symbols: ● and ■, activity in the absence of the 100-kDa receptor; ○ and □, activity in the presence of 500 fmol of the 100-kDa receptor. For the experiments whose results are shown in panel A, the EGF–Affi-Gel-purified 170-kDa receptor (70 fmol) was incubated at 4°C for 10 min with or without the 100-kDa peptide (500 fmol) and then incubated at 20°C for 2 min with [γ-^{32}P]ATP (final concentration, 0.3 to 30 nM) and 1 mM $MnCl_2$ as described in the legend to Fig. 2. Our previous studies (7) showed that the autokinase reaction is linear for up to 2 min at 20°C. After termination of the reaction, the mixtures were subjected to electrophoresis and autoradiography. The extent of receptor phosphorylation was determined by measuring the radioactivity in the region of the dried gel containing the ^{32}P-labeled receptor band. Dried gel strips of similar dimensions from adjacent regions were also measured to correct for background radioactivity. For the experiment whose results are shown in panel B, the EGF–Affi-Gel-purified 170-kDa receptor was treated with the 100-kDa peptide and EGF as described in the legend to Fig. 2. To the 15-μl mixture was added 2 μl of a tridecapeptide substrate solution (5, 7, 19) to yield a final concentration of 0.5 to 3 mM peptide after the addition of ATP. Phosphorylation was initiated at 20°C by addition of 5 μl of 60 μM [γ-^{32}P]ATP containing 4 mM $MnCl_2$. After incubation at 20°C for 5 min, the reaction was terminated and the mixture was analyzed as previously described (7).

of related growth factor receptors. To test whether the inhibition is specific for the EGF receptor, we studied two other receptor kinases—the PDGF receptor kinase and the insulin receptor kinase. These are transmembrane proteins belonging to the tyrosine kinase family that display ligand-stimulated autophosphorylating ability (9). The 100-kDa protein had no effect on either basal or ligand-stimulated autokinase activities of these receptors (Fig. 6). Other studies (K. Kokai, M. Greene, and M. Das, unpublished data) have shown that the 100-kDa protein has no effect on the autokinase activity of the neu protein, a transmembrane receptorlike entity which is unique in its external domain but shows extensive homology with the EGF receptor in its kinase domain (1). These results demonstrate that the kinase-inhibitory effect of the 100-kDa protein is specific for the EGF receptor.

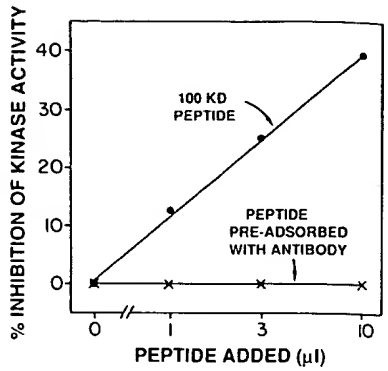

FIG. 5. The inhibition of kinase was due to the immunoreactive 100-kDa receptor. A solution of the 100-kDa receptor (5 pmol in 100 μl of 20 mM HEPES [pH 7.4]–10% glycerol–10 μg of leupeptin per ml–2 mM PMSF–0.2 mM sodium vanadate) was incubated at 4°C for 2 h with 20 μg of purified mouse monoclonal antibody 425 (16). The antigen-antibody complex and any free antibody were removed by two successive treatments with goat anti-mouse immunoglobulin G-agarose (binds 0.5 mg of mouse immunoglobulin G per ml of packed gel). Each treatment involved stirring at 4°C for 1.5 h with 50 μl of packed agarose. The final supernatant fraction was used as the preadsorbed preparation. The control 100-kDa protein preparation received no monoclonal antibody 425. Both preparations were tested for inhibition of phosphorylation of a synthetic peptide substrate by the EGF–Affi-Gel-purified 170-kDa receptor (70 fmol) as described in the legend to Fig. 4.

The 100-kDa receptor may specifically associate with the 170-kDa EGF receptor. The results suggest that the 100-kDa receptor may inhibit the EGF receptor kinase by specifically associating with it. This possibility was tested as follows.

Mixtures of the 100-kDa and 170-kDa receptors were cross-linked by using 0.5 mM 3,3′-bis(sulfosuccinimido) suberate, a noncleavable chemical cross-linking reagent, and then analyzed by reducing SDS-PAGE and immunoblotting with a specific anti-EGF receptor antibody (17). The results (Fig. 7, lane 2) showed the formation of a covalent cross-linked complex of 250 kDa. This cross-linked complex was not seen in experiments with the 100-kDa receptor alone (data not shown), indicating the absence of homodimers. The 250-kDa cross-linked complex (Fig. 7, lane 2) was not seen in experiments in which the 170-kDa receptor was pretreated with EGF and then incubated with the 100-kDa receptor (Fig. 7, lane 1). Pretreatment of the intact EGF receptor with EGF was also effective in blocking the ability of the 100-kDa receptor to inhibit its kinase function (Fig. 8).

We next tested whether the 250-kDa cross-linked complex (Fig. 7, lane 2) represents a heterodimer of the 100-kDa and 170-kDa receptors. Mixtures of the 100-kDa and 170-kDa receptors were cross-linked by using a cleavable cross-linker, 3,3′-dithiobis(sulfosuccinimido)propionate and then subjected to nonreducing SDS-PAGE and immunoblotting (Fig. 7, lane 3). It should be noted that the antibody used for immunoblotting bound poorly to unreduced receptor forms: i.e., reactivity with the unreduced 170-kDa receptor was

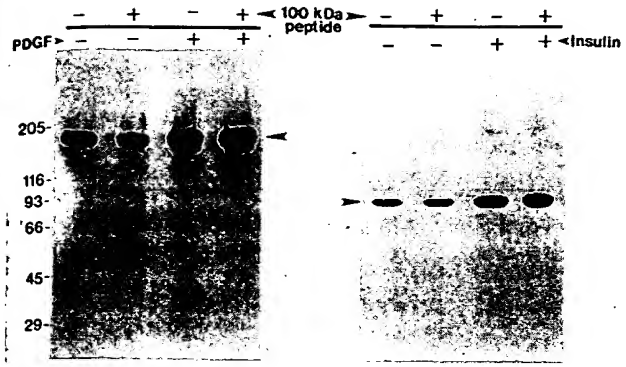

FIG. 6. The 100-kDa receptor did not inhibit the activity of PDGF receptor kinase or insulin receptor kinase. The 100-kDa peptide (2.5 pmol) was incubated at 4°C for 10 min with a purified preparation of either the PDGF receptor (~20 fmol) or the insulin receptor (~70 fmol) in a total volume of 15 μl under conditions identical to those described in the legend to Fig. 2 for the EGF receptor. The mixtures were then incubated at 4°C for an additional 10 min in the presence or absence of either 1 μl of 70 nM PDGF (for the reaction mixtures containing the PDGF receptor) or 1 μl of 40 μM insulin (for the reactions mixtures containing the insulin receptor). Phosphorylation was initiated at 20°C by addition of 5 μl of 60 μM [γ-^{32}P]ATP containing 8 mM $MgCl_2$ and 2 mM $MnCl_2$ to the tubes containing the PDGF receptor or 5 μl of 60 μM [γ-^{32}P]ATP containing 4 mM $MnCl_2$ to the tubes containing the insulin receptor. After incubation at 20°C for 15 min, the reactions were terminated and the mixtures were analyzed by reducing SDS-PAGE and autoradiography as described in the legend to Fig. 2. The numbers to the left indicate molecular size in kilodaltons. The arrowheads in the center indicate the positions of PDGF receptor and the insulin receptor β subunit.

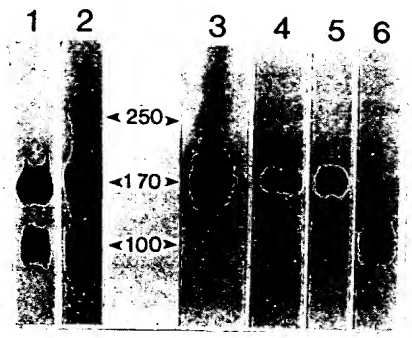

FIG. 7. Specific association of the 100-kDa receptor with the intact EGF receptor. The EGF–Affi-Gel-purified 170-kDa receptor (1 pmol) was preincubated at 20°C for 5 min in 75 μl of HEPES-glycerol-Triton buffer containing either 2 μM EGF (lane 1) or no EGF (lanes 2 and 3 plus an additional sample). The mixtures were then incubated at 4°C for 15 min with the 100-kDa receptor (5 pmol) in a total volume of 100 μl. Chemical cross-linking was initiated by addition of 10 μl of 5 mM 3,3′-bis(sulfosuccinimido)suberate (lanes 1 and 2) or 3,3′-dithiobis(sulfosuccinimido)propionate (lane 3 plus a duplicate). After incubation at 4°C for 10 min, excess cross-linking was quenched by addition of 10 μl of 1 M ethanolamine (pH 8.0). The mixtures were then subjected to SDS-PAGE in 3.5 to 10% gradient acrylamide gels under reducing conditions [for 3,3′-bis(sulfosuccinimido)suberate-cross-linked samples] (lanes 1 and 2 or nonreducing conditions [for 3,3′-dithiobis(sulfosuccinimido)propionate-treated samples] (lane 3 plus a duplicate). The electrophoretograms were either analyzed by immunoblotting with an external-domain-specific anti-EGF receptor polyclonal antibody (lanes 1 to 3) or subjected to the following treatment [for the duplicate 3,3′-dithiobis(succinimido)propionate-treated sample]. The region of the unfixed wet gel corresponding to the 250-kDa cross-linking complex and the regions where the free receptors (170 and 100 kDa) are expected were cut out, stirred at 37°C for 10 h with SDS sample buffer containing β-mercaptoethanol, and then subjected to SDS-PAGE and immunoblotting (lanes 4 to 6). It should be noted that the antibody used for immunoblotting did not bind to the unreduced 100-kDa receptor and bound only poorly to the unreduced 170-kDa receptor (see the text).

~30% of that with the reduced form, and there was no detectable reactivity with the unreduced 100-kDa receptor. Because of this low antibody reactivity, the poor efficiency of cross-linking, and the lack of sharpness of separation in nonreducing gels, the amount of the 250-kDa complex detected by this method was small. Reduction of the 250-kDa complex, followed by reducing SDS-PAGE and immunoblotting, showed the presence of both the 100-kDa and 170-kDa receptors (Fig. 7, lane 4). The difference in the intensity of the 170-kDa and 100-kDa bands in lane 4 reflects a difference in immunoreactivity; i.e., the polyclonal anti-EGF receptor antibody showed higher reactivity with the intact receptor than with the 100-kDa truncated form. Overall, the results suggest that the 100-kDa receptor may form a heterodimer with the intact receptor.

It should be noted that the free 100-kDa protein was not immunodetectable in the nonreducing gel (Fig. 7, lane 3) because of the characteristic mentioned in a previous paragraph—the unreduced 100-kDa receptor showed no binding to the polyclonal anti-EGF receptor antibody in immunoblots. Proof of the presence of the free 100-kDa receptor in the expected region of the gel is shown in Fig. 7, lane 6. Reduction of the gel piece, followed by SDS-PAGE and immunoblotting, revealed the presence of the immunoreactive 100-kDa receptor.

DISCUSSION

The results described here can be summarized as follows. (i) A 100-kDa EGF-binding receptor form inhibited the tyrosine kinase activity of the intact 170-kDa EGF receptor. (ii) Antibody preadsorption and other studies demonstrated that the inhibition was due to the immunoreactive 100-kDa EGF receptor and not to a contaminating phosphatase or protease. (iii) The 100-kDa receptor was not a substrate for the receptor kinase and had no effect on the affinity of the receptor kinase for its substrates. These results indicate that the inhibition was not due to competition for the kinase substrate site. (iv) The inhibition was not due to a reduction in the available EGF, because the 100-kDa receptor was an effective inhibitor of both basal kinase and EGF-stimulable kinase; moreover, under the experimental condition used, there was a high excess of EGF over the EGF-binding receptor forms. (v) The 100-kDa receptor did not inhibit the activities of related receptor kinases, which are homologous to the EGF receptor in their kinase domains but are distinctive in their external domains. These results suggest a role for the external domain in determining target specificity for inhibition. (vi) A specific interaction of the 100-kDa receptor with the intact 170-kDa EGF receptor was suggested by chemical cross-linking analysis. These results suggest a specific association of the EGF-binding 100-kDa protein with the structurally unique EGF-binding domain of the EGF receptor kinase.

Growth factor receptors belong to a unique class of bipolar multisited proteins. They have intrinsic tyrosine kinase catalytic sites built into their large cytoplasmic domains. These catalytic proteins are capable of self-regulation and

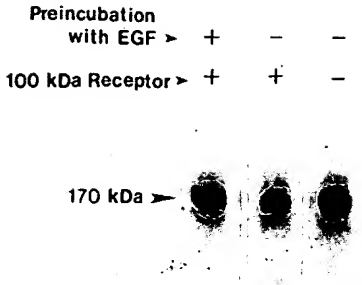

FIG. 8. Preincubation with EGF blocked the kinase-inhibitory activity of the 100-kDa protein. The EGF–Affi-Gel-purified 170-kDa receptor (70 fmol) was preincubated at 20°C for 5 min with or without 2 μM EGF in 15 μl of HEPES-glycerol buffer (see Fig. 2, legend) and then incubated at 20°C for 5 min with or without the 100-kDa receptor (250 fmol) in a total volume of 20 μl. EGF was then added to the tubes which had not received EGF before (the final concentration of EGF in all of the tubes was 1.2 μM; the final volume was 25 μl), and the incubation was continued at 20°C for another 5 min. Phosphorylation was initiated by addition of 15 μl of a solution containing 60 μM [γ-^{32}P]ATP–4 mM $MnCl_2$. After incubation at 20°C for 5 min, the phosphorylated products were analyzed by electrophoresis and autoradiography as described in the legend to Fig. 2.

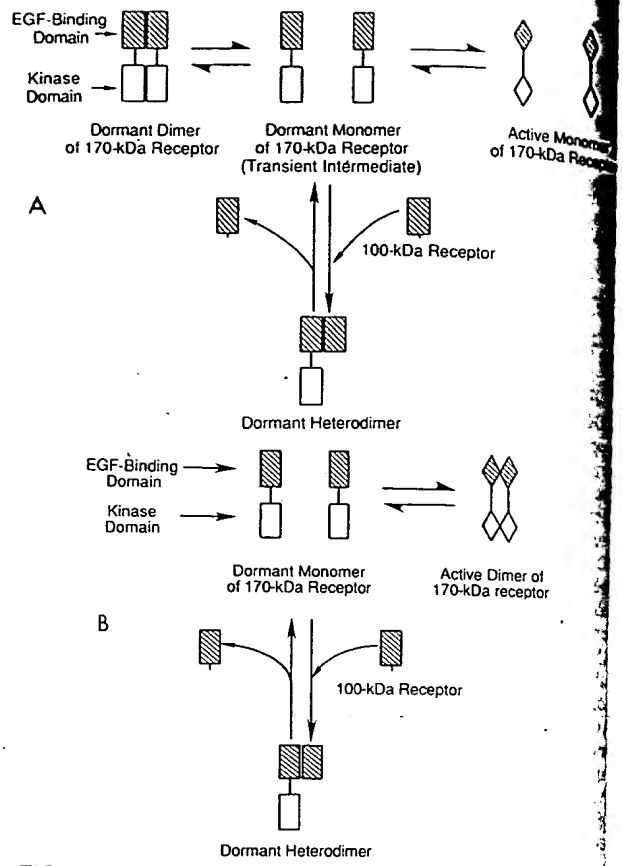

FIG. 9. Models for possible association-dissociation events between intact and truncated EGF receptor forms. (A) Inhibition of kinase due to prevention of active monomer formation. (B) Inhibition of kinase due to prevention of active dimer formation. The bases for these two conflicting models are discussed in the text.

are activated only upon growth factor binding to their external domains. It is an open question how this self-regulation of kinase is achieved and how growth factor binding abolishes the regulation and activates the kinase. Two recent studies (14, 18) suggest that the kinase site in the monomeric EGF receptor can be activated independently of interreceptor association, suggesting a role for the single membrane-spanning segment in signal transduction. This may not be unlikely in light of the known role of Val→Glu transmembrane mutation in the constitutive activation of neu kinase activity (1). However, other studies on receptor kinases suggest that the kinase site may be controlled by a dynamic equilibrium between monomeric and dimeric receptors (5, 7, 8, 24). The intact EGF receptor is known to form dimers, and the inability to detect dimers of truncated forms that lack either the kinase or the EGF-binding domain implies that integrity of the receptor molecule is essential for dimerization (5). In light of these observations, it is particularly pertinent that the 100-kDa soluble receptor is capable of modulating the kinase function of the intact 170-kDa receptor.

Our past and present results suggest the following type of association-dissociation behavior (Fig. 9A). A dormant monomeric form of the 170-kDa receptor may serve as a transient intermediate between the active monomer and the dormant dimer. The 100-kDa receptor may interact specifically with this transient species and stabilize the state of dormancy. Heterodimer formation and half-maximal inhibition of receptor kinase activity were seen only at three- to fivefold excess of the 100-kDa receptor over the intact receptor form. Thus, the 170-kDa receptor is likely to form a heterodimer with the 100-kDa truncated receptor when there is an excess of the truncated receptor.

Our findings on receptor kinase regulation through association contradict those of other workers who found that receptor associations are stimulatory (8, 24). According to the association-dependent activation model (Fig. 9B), the results described here could also be interpreted as suggesting that the 100-kDa receptor competes with the intact receptor for self-association.

Little is known about the chemistry of interreceptor interaction in the external domain. Interchain disulfide bonds and other covalent bonds are not involved in the case of the EGF receptor. In the major histocompatibility complex antigen interaction system (which can be considered as a ligand-receptor system) there is some evidence for the following type of association mechanism (reviewed in reference 21). There is an integral site within the major histocompatibility complex (analog of a receptor) that is homologous to the antigen (analog of a ligand) which binds to a complementary site in the major histocompatibility complex, resulting in isologous interreceptor association. The free antigen (the ligand) would be able to displace the integral segment in the major histocompatibility complex (the receptor) and prevent interreceptor association. According to this model the biochemical basis for interreceptor interaction lies in structural motifs within the receptor external domain that correspond to the ligand and the ligand-binding site. It remains to be tested whether a similar mechanism control the association-dissociation behavior of the EGF receptor

It is of interest to compare the properties of the soluble EGF receptor with those of soluble CD4, a truncated protein derived from the cellular receptor for human immunodeficiency virus type I (reviewed in reference 23). Both the soluble EGF receptor and the soluble CD4 receptor can bind to EGF and gp120, respectively, and can block the activities of intact receptors when present in excess. Competition for ligand binding is one of the possible explanations for this blocking behavior. The other possibility is the association of the intact receptor with truncated forms, leading to negative regulation of receptor function.

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